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(54) Title: MODULATION OF SECONDARY METABOLITE PRODUCTION BY ZINC BINUCLEAR CLUSTER PROTEINS

(57) Abstract: Disclosed are methods for the modulation of secondary metabolite production by fungi through genetic manipulation of such fungi. Also disclosed are new commercial processes using ZBC proteins, or variants thereof, to significantly increase useful secondary metabolite production. Generally, the methods according to the invention comprise expressing in a fungus a ZBC protein or a variant thereof.

WO 02/24865 A2

## MODULATION OF SECONDARY METABOLITE PRODUCTION BY ZINC BINUCLEAR CLUSTER PROTEINS

### BACKGROUND OF THE INVENTION

#### Field of the Invention

The invention relates to secondary metabolite production by fungi. More particularly, the invention relates to modulation of secondary metabolite production by fungi through genetic manipulation of such fungi.

#### Description of the Related Art

Secondary metabolite production by various fungi has been an extremely important source of a variety of therapeutically significant pharmaceuticals.  $\beta$ -lactam antibacterials such as penicillin and cephalosporin are produced by *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively, and these compounds are by far the most frequently used antibacterials (reviewed in Luengo and Penalva (1994), *Prog. Ind. Microbiol.* 29: 603-38; Jensen and Demain (1995), *Biotechnology* 28: 239-68; Brakhage (1998), *Microbiol. Mol. Biol. Rev.* 62: 547-85). Cyclosporin A, a member of a class of cyclic undecapeptides, is produced by *Tolypocladium inflatum*. Cyclosporin A dramatically reduces morbidity and increases survival rates in transplant patients (Borel (1986), *Prog. Allergy* 38: 9-18). In addition, several fungal secondary metabolites are cholesterol lowering drugs, including lovastatin, which is made by *Aspergillus terreus* and several other fungi (Alberts *et al.* (1980), *Proc. Natl. Acad. Sci. USA* 77: 3957-3961). These and many other fungal secondary metabolites have contributed greatly to health care throughout the world (see Demain (1992), *Ciba Found. Symp.* 171: 3-16; Bentley (1991), *Crit. Rev. Biotechnol.* 19: 1-40).

Unfortunately, many challenges are encountered between the detection of a secondary metabolite activity to production of significant quantities of pure drug. Thus, efforts have been made to improve the production of secondary metabolites by fungi. Some of these efforts have attempted to improve production by modification of the growth medium or the bioreactor used to carry out the fermentation. Buckland *et al.* (1989), in *Topics in Industrial Microbiology: Novel Microbial Products for*

*Medicine and Agriculture*, Elsevier, Amsterdam, pp. 161-169, discloses improved lovastatin production by modification of carbon source and also teaches the superiority of a hydrofoil axial flow impeller in the bioreactor. Other efforts have involved strain improvements, either through re-isolation or random mutagenesis. Agathos *et al.* (1986), *J. Ind. Microbiol.* 1: 39-48, teaches that strain improvement and process development together resulted in a ten-fold increase in cyclosporin A production. While important, studies of these types have still left much room for improvement in the production of secondary metabolites.

More recently, strains have been improved by manipulation of the genes encoding the biosynthetic enzymes that catalyze the reactions required for production of secondary metabolites. Penalva *et al.* (1998), *Trends Biotechnol.* 16: 483-489 discloses that production strains of *P. chrysogenum* have increased copy number of the penicillin synthesis structural genes. Other studies have modulated expression of other biosynthetic enzyme-encoding genes, thereby affecting overall metabolism in the fungus. Mingo *et al.* (1999), *J. Biol. Chem.* 274: 14545-14550, demonstrate that disruption of *phacA*, an enzyme in *A. nidulans* that catalyzes phenylacetate 2-hydroxylation, leads to increased penicillin production, probably by elimination of competition for the substrate phenylacetate. Similarly, disruption of the gene encoding aminoadipate reductase in *P. chrysogenum* increased penicillin production, presumably by eliminating competition for the substrate alpha-aminoadipate (Casquero *et al.* (1999), *J. Bacteriol.* 181: 1181-1188).

Thus, genetic manipulation holds promise for improving production of secondary metabolites. Genetic manipulation to increase the activity of biosynthetic enzymes for secondary metabolite production or to decrease the activity of competing biosynthetic pathways has proven effective for improving production. Maximum benefit can be achieved by combining several strategies of manipulation. For example, modulating the expression of genes that regulate the biosynthetic enzyme-encoding genes can improve production. In addition, genetic manipulation can be used to impact upon the challenges that are encountered in the fermentor run or downstream processing (*e.g.*, energy cost, specific production of desired metabolite, maximal recovery of metabolite, cost of processing waste from fermentations). There is, therefore, a need for methods for improving secondary metabolite production in a

fungus, comprising modulating the expression of a gene involved in regulation of secondary metabolite production in fungi.

One challenge is to identify the types of genes that would be useful for such modulation. Todd and Andrianopoulos (1997), *Fungal Genetics and Biology* 21: 388-405, teaches that Zn(II)<sub>2</sub>Cys<sub>6</sub> proteins (zinc binuclear cluster proteins, or "ZBC proteins") are involved in a wide range of processes, including primary and secondary metabolism. This reference teaches that such proteins are primarily, though not exclusively, transcriptional activators. Chang *et al.* (1995), *Applied Environ. Microbiol.* 61: 2372-2377, teaches that increased expression of aflR, a ZBC protein, relieves nitrate inhibition of aflatoxin biosynthesis in *Aspergillus parasiticus*. PCT Publication WO 00/37629, teaches that over-expressing lovE, another ZBC protein, increases lovastatin production in *Aspergillus terreus*. Noel *et al.* (1998), *Mol. Microbiol.* 27: 131-142, teaches that xlnR, a ZBC protein, induces expression of xylanolytic extracellular enzymes in *Aspergillus niger*. Hasper *et al.* (2000), *Mol. Microbiol.* 36: 193-200, teaches that xlnR also regulates D-xylose reductase gene expression in *Aspergillus niger*. D'Alessio and Brandriss (2000), *J. Bacteriology* 182: 3748-3753, discloses that Gal4p, a ZBC protein, can activate the *PUT* (proline utilization) genes in a *Saccharomyces cerevisiae* strain lacking the normal gene for regulation of this pathway, *PUT3*. PCT Publication WO 00/20596 discloses that prtT, a ZBC protein, activates extracellular proteases in *Aspergillus niger*.

Numerous studies have examined the effects of mutations in genes that encode ZBC proteins. Crowley *et al.* (1998), *J. Bacteriol.* 180: 4177-4183, discloses that a single missense mutation in *UPC2*, a ZBC gene, results in pleiotropic effects in *Saccharomyces cerevisiae*. Friden *et al.* (1989), *Mol. Cell. Biol.* 9: 4056-4060, teaches that a large internal deletion in *Leu3p*, a ZBC protein, in *Saccharomyces cerevisiae* causes the protein to be a constitutive transcriptional activator. Oestreicher and Scazzocchio (1995), *J. Mol. Biol.* 249: 693-699, discloses that a single amino acid change in *Yc462*, a ZBC protein, leads to constitutive, hyperinducible and derepressed expression of at least three genes in *Aspergillus nidulans*. Wang *et al.* (1999), *J. Biol. Chem.* 274: 19017-19024, discloses that nine distinct missense mutations in *LEU3* affect the masking of the activation domain of that ZBC protein. Dickson *et al.* (1990), *Nucleic Acids Res.* 18: 5213-5217, discloses that single amino acid changes in

the C terminal region of Gal4p and Lac9p, two ZBC proteins, lead to constitutive expression of target genes. Marczak and Brandriss (1991), Mol. Cell. Biol. 11: 2609-2619, teaches that single point mutations in *PUT3*, a ZBC gene from *Saccharomyces cerevisiae*, lead to either constitutive or uninducible expression of proline utilization genes. Carvajal *et al.* (1997), Mol. Gen. Genet. 256: 406-415, teaches that single amino acid substitutions in Pdr1p, a ZBC protein from *Saccharomyces cerevisiae*, are responsible for over-expression of three transporter genes associated with multiple drug resistance. Nourani *et al.* (1997), Mol. Gen. Genet. 256: 397-405, teaches that substitutions in a conserved region of Pdr3p, a ZBC protein from *Saccharomyces cerevisiae*, leads to gain of function mutations. Zhou *et al.* (1990), Nucleic Acids Res. 18: 291-298, discloses that deletion of all or part of the linker region of Leu3p results in unmodulated activation of Leu3p target genes. Herlich *et al.* (1998), Fungal Genetics Biol. 23: 1807-1845, teaches that deletion of three amino acids in the C terminus of AflR results in increased expression of the aflatoxin pathway.

These studies demonstrate that ZBC genes can be manipulated in beneficial ways and may have promise as regulators of secondary metabolism. Unfortunately, no one has been able to create a commercial process in which production of a useful secondary metabolite has been significantly increased through the action of a ZBC protein. There is, therefore, a need for new commercial processes using ZBC proteins, or variants thereof, to significantly increase useful secondary metabolite production.

### **SUMMARY OF THE INVENTION**

The invention relates to secondary metabolite production by fungi. More particularly, the invention relates to modulation of secondary metabolite production by fungi through genetic manipulation of such fungi. The invention provides new commercial processes using ZBC proteins, or variants thereof, to significantly improve the production of useful secondary metabolites. Generally, the methods according to the invention comprise expressing in a fungus a ZBC protein or a variant thereof.

In a first aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite produced by the fungus. The methods according to this aspect of the invention

comprise modulating the expression of a ZBC gene or gene variant in a manner that improves the yield of the secondary metabolite.

In a second aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite in the fungus, the methods comprising modulating the expression of a ZBC gene or gene variant in a manner that improves the productivity of the secondary metabolite.

In a third aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing efflux or excretion of the secondary metabolite, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that increases efflux or excretion of the secondary metabolite.

In a fourth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by decreasing production of side products or non-desired secondary metabolites, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that decreases production of side products or non-desired secondary metabolites.

In a fifth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that alters the characteristics of the fungus.

In a sixth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that causes conditional lysis.

In a seventh aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite made by the same organism, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that increases resistance to the deleterious effects of exposure to a secondary metabolite.

In an eighth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by modulating the expression of one or more genes, the method comprising modulating the expression of a ZBC gene or gene variant that does not normally modulate the expression of such gene or genes.

In a ninth aspect, the invention provides genetically modified fungi, wherein the genetically modified fungi have an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods according to the invention.

In a tenth aspect, the invention provides a method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to the invention under conditions suitable for the production of secondary metabolites.

### **DETAILED DESCRIPTION**

The invention relates to secondary metabolite production by fungi. More particularly, the invention relates to modulation of secondary metabolite production by fungi through genetic manipulation of such fungi. All issued patents, published and pending patent applications, and other references cited herein reflect the level of knowledge in this field and are hereby incorporated by reference in their entirety. In case of any conflict between the teachings of a cited reference and this specification, the latter shall prevail.

The invention provides new commercial processes using ZBC proteins, or variants thereof, to significantly increase useful secondary metabolite production. Generally, the methods according to the invention comprise expressing in a fungus a ZBC protein or a variant thereof. All aspects of the invention contemplate the modulation of one or more ZBC genes in a fungal cell of interest.

In a first aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite produced by the fungus. The methods according to this aspect of the invention comprise modulating the expression of a ZBC gene or gene variant in a manner that improves the yield of the secondary metabolite.

As used for all aspects of the invention, the term "improving production of a secondary metabolite" means positively impacting one or more of the variables that

affect the process of production of secondary metabolites in a fungal fermentation. These variables include, without limitation, the amount of secondary metabolite being produced, the volume required for production of sufficient quantities, the cost of raw materials and energy, the time of fermentor run, the amount of waste that must be processed after a fermentor run, the specific production of the desired metabolite, the percent of produced secondary metabolite that can be recovered from the fermentation broth, and the resistance of an organism producing a secondary metabolite to possible deleterious effects of contact with the secondary metabolite. Also for all aspects, the term "secondary metabolite" means a compound, derived from primary metabolites, that is produced by an organism, is not a primary metabolite, is not ethanol or a fusel alcohol, and is not required for growth under standard conditions. Secondary metabolites are derived from intermediates of many pathways of primary metabolism. These pathways include, without limitation, pathways for biosynthesis of amino acids, the shikimic acid pathway for biosynthesis of aromatic amino acids, the polyketide biosynthetic pathway from acetyl coenzyme A (CoA), the mevalonic acid pathway from acetyl CoA, and pathways for biosynthesis of polysaccharides and peptidopolysaccharides. Secondary metabolism involves all primary pathways of carbon metabolism (*Fungal Physiology*, Chapter 9, pp 246-274, Griffin (ed.), John Wiley & Sons, Inc., New York, (1994)). "Secondary metabolites" also include intermediate compounds in the biosynthetic pathway for a secondary metabolite that are dedicated to the pathway for synthesis of the secondary metabolite. "Dedicated to the pathway for synthesis of the secondary metabolite" means that once the intermediate is synthesized by the cell, the cell will not convert the intermediate to a primary metabolite. "Intermediate compounds" also include secondary metabolite intermediate compounds which can be converted to useful compounds by subsequent chemical conversion or subsequent biotransformation. Nevertheless, providing improved availability of such intermediate compounds still leads to improved production of the ultimate useful compound, which itself may be referred to herein as a secondary metabolite. The yeast *Saccharomyces cerevisiae* is not known to produce secondary metabolites. The term "primary metabolite" means a natural product that has an obvious role in the functioning of the relevant organism. Primary metabolites include, without limitation, compounds involved in the biosynthesis of lipids,



carbohydrates, proteins, and nucleic acids. The term "increasing the yield of the secondary metabolite" means increasing the quantity of the secondary metabolite present in the fermentation broth per unit volume of fermentation broth.

The term "ZBC gene" means any gene encoding a protein having as part of its structure Cys-(Xaa)<sub>2</sub>-Cys-(Xaa)<sub>6</sub>-Cys-(Xaa)<sub>5-16</sub>-Cys-(Xaa)<sub>2</sub>-Cys-(Xaa)<sub>6-8</sub>-Cys (see e.g., Todd and Andrianopoulos (1997), *Fungal Genetics and Biology* 21: 388-405), wherein Xaa is any amino acid, each of which can be the same or different. Preferred ZBC genes according to this aspect of the invention include, without limitation, those genes identified in Table 1, below, and any fungal homologs thereof.

**TABLE 1**  
**Examples of preferred ZBC genes**

Name	Length (aa)	Size (bp)	Organism	SEQ ID NO
AAB05250_Hh	455	1365	<i>Fusarium solani</i>	13
AAC98670_Ca	517	1551	<i>Candida albicans</i>	15
AC15_Nc	865	2595	<i>Neurospora crassa</i>	17
acr2_Nc	595	1785	<i>Neurospora crassa</i>	19
AF168613_4_Ap	491	1473	<i>Aspergillus parasiticus</i>	21
AF230811_1_Pg	974	2922	<i>Pyricularia grisea</i>	23
aflR_Af	437	1311	<i>Aspergillus flavus</i>	25
AFLR_An	433	1299	<i>Aspergillus nidulans</i>	27
aflR_Ao	384	1152	<i>Aspergillus oryzae</i>	29
aflR_Ap	444	1332	<i>Aspergillus parasiticus</i>	31
alcR_An	821	2463	<i>Aspergillus nidulans</i>	33
AmdR-An	765	2295	<i>Aspergillus nidulans</i>	35
AmdR-Ao	735	2205	<i>Aspergillus oryzae</i>	37
AmyR-Anig	579	1737	<i>Aspergillus niger</i>	39
amyRAn	662	1986	<i>Aspergillus nidulans</i>	41
amyRAo	604	1812	<i>Aspergillus oryzae</i>	43
An13_An	311	933	<i>Aspergillus nidulans</i>	45
ARG81_YEAST	880	2640	<i>Saccharomyces cerevisiae</i>	47
ARGRII_YEAST	879	2637	<i>Saccharomyces cerevisiae</i>	49
At18_At	397	1191	<i>Aspergillus terreus</i>	51
BAA21449_Sp	738	2214	<i>Schizosaccharomyces pombe</i>	53
BAA87112_Sp	71	212	<i>Schizosaccharomyces pombe</i>	55
BAA87304_Sp	188	564	<i>Schizosaccharomyces</i>	57

			<i>pombe</i>	
C23788_Pa	412	1236	<i>Pichia anomala</i>	59
CAA11231_Pa	529	1587	<i>Pichia anomala</i>	61
CAA18305_Sp	867	2601	<i>Schizosaccharomyces</i>	63
			<i>pombe</i>	
CAA18884_Sp	397	1191	<i>Schizosaccharomyces</i>	65
			<i>pombe</i>	
CAA19035_Sp	827	2481	<i>Schizosaccharomyces</i>	67
			<i>pombe</i>	
CAA19036_Sp	560	1680	<i>Schizosaccharomyces</i>	69
			<i>pombe</i>	
CAA19070_Sp	525	1575	<i>Schizosaccharomyces</i>	71
			<i>pombe</i>	
CAA19171_Sp	743	2229	<i>Schizosaccharomyces</i>	73
			<i>pombe</i>	
CAA 19174_Sp	815	2445	<i>Schizosaccharomyces</i>	75
			<i>pombe</i>	
CAA20477_Sp	547	1641	<i>Schizosaccharomyces</i>	77
			<i>pombe</i>	
CAA21815_Sp	857	2571	<i>Schizosaccharomyces</i>	79
			<i>pombe</i>	
CAA21917_Sp	594	1782	<i>Schizosaccharomyces</i>	81
			<i>pombe</i>	
CAA21921_Sp	595	1785	<i>Schizosaccharomyces</i>	83
			<i>pombe</i>	
CAA21933_Ca	510	1530	<i>Candida albicans</i>	85
CAA22445_Sp	480	1440	<i>Schizosaccharomyces</i>	87
			<i>pombe</i>	
CAA22655_Sp	767	2301	<i>Schizosaccharomyces</i>	89
			<i>pombe</i>	
CAA22853_Sp	736	2208	<i>Schizosaccharomyces</i>	91
			<i>pombe</i>	
CAA92308_Sp	603	1809	<i>Schizosaccharomyces</i>	93
			<i>pombe</i>	
CAB16735_Sp	783	2349	<i>Schizosaccharomyces</i>	95
			<i>pombe</i>	
CAB52588_Sm	689	2067	<i>Sordaria macrospora</i>	97
CAB59617_Sp	625	1875	<i>Schizosaccharomyces</i>	99
			<i>pombe</i>	
CAB61777_Sp	654	1962	<i>Schizosaccharomyces</i>	101
			<i>pombe</i>	
CAB71797_Toly poclad	905	2715	<i>Tolypocladium inflatum</i>	103
CAR80_YEAST	836	2508	<i>Saccharomyces</i>	105
			<i>cerevisiae</i>	
CAT8_Ca	1056	3168	<i>Candida albicans</i>	107
CAT8_Kl	1445	4335	<i>Kluyveromyces lactis</i>	109
CAT8_YEAST	1433	4299	<i>Saccharomyces</i>	111

CEP3_YEAST	608	1824	<i>cerevisiae</i>	
			<i>Saccharomyces</i>	113
CHA4_YEAST	648	1944	<i>cerevisiae</i>	
			<i>Saccharomyces</i>	115
CMR1_CI	984	2952	<i>cerevisiae</i>	
			<i>Colletotrichum</i>	117
CT1A_Fs	909	2727	<i>lagenarium</i>	
			<i>Haematonectria</i>	119
CT1B_Fs	882	2646	<i>haematococca</i>	
			<i>Haematonectria</i>	121
			<i>haematococca</i>	
CZF1_Ca	388	1164	<i>Candida albicans</i>	123
DAL81_YEAST	970	2910	<i>Saccharomyces</i>	125
			<i>cerevisiae</i>	
ECM22_YEAST	814	2442	<i>Saccharomyces</i>	127
			<i>cerevisiae</i>	
FacB_An	867	2601	<i>Aspergillus nidulans</i>	129
FacB_Anig	862	2586	<i>Aspergillus niger</i>	131
FacB_Ao	859	2577	<i>Aspergillus oryzae</i>	133
FLUF_Nc	792	2376	<i>Neurospora crassa</i>	135
GAL4_YEAST	881	2643	<i>Saccharomyces</i>	137
			<i>cerevisiae</i>	
HAL9_YEAST	1030	3090	<i>Saccharomyces</i>	139
			<i>cerevisiae</i>	
HAP1_1483YEA ST	1483	4449	<i>Saccharomyces</i>	141
			<i>cerevisiae</i>	
HAP1_1502YEA ST	1502	4506	<i>Saccharomyces</i>	143
			<i>cerevisiae</i>	
LAC9_Kl	865	2595	<i>Kluyveromyces lactis</i>	145
lac9_Kmarx	865	2595	<i>Kluyveromyces</i>	147
			<i>marxianus var. lactis</i>	
LEU3_YEAST	886	2658	<i>Saccharomyces</i>	149
			<i>cerevisiae</i>	
lovE_At	503	1509	<i>Aspergillus terreus</i>	151
lovEv2_At	469	1492	<i>Aspergillus terreus</i>	153
lovU_At	742	2226	<i>Aspergillus terreus</i>	155
LYS14_YEAST	790	2370	<i>Saccharomyces</i>	157
			<i>cerevisiae</i>	
M81157_1_Spast or	470	1410	<i>Saccharomyces</i>	159
			<i>pastorianus</i>	
MAL13_YEAST	473	1419	<i>Saccharomyces</i>	161
			<i>cerevisiae</i>	
MAL23_YEAST	470	1410	<i>Saccharomyces</i>	163
			<i>cerevisiae</i>	
MAL33_YEAST	468	1404	<i>Saccharomyces</i>	165
			<i>cerevisiae</i>	
MAL63_YEAST	470	1410	<i>Saccharomyces</i>	167
			<i>cerevisiae</i>	

MAL6_Scarlsberg	473	1419	<i>Saccharomyces carlsbergensis</i>	169
MSP8_YEAST	1429	4287	<i>Saccharomyces cerevisiae</i>	171
NIRA_An	892	2676	<i>Aspergillus nidulans</i>	173
NTT4_Nc	1090	3270	<i>Neurospora crassa</i>	175
ntf1/thi1_Sp	775	2325	<i>Schizosaccharomyces pombe</i>	177
OAF1_YEAST	1062	3186	<i>Saccharomyces cerevisiae</i>	179
PDR3_YEAST	976	2928	<i>Saccharomyces cerevisiae</i>	181
PIP2_YEAST	996	2988	<i>Saccharomyces cerevisiae</i>	183
PPR1_YEAST	904	2712	<i>Saccharomyces cerevisiae</i>	185
PRIB_Le	565	1695	<i>Shiitake mushroom</i>	187
prnA_An	818	2454	<i>Aspergillus nidulans</i>	189
PUT3_YEAST	979	2937	<i>Saccharomyces cerevisiae</i>	191
QA1F_Nc	816	2448	<i>Neurospora crassa</i>	193
QUTA_An	825	2475	<i>Aspergillus nidulans</i>	195
RGT1_YEAST	1170	3510	<i>Saccharomyces cerevisiae</i>	197
SEF1_K1	1071	3213	<i>Kluyveromyces lactis</i>	199
SEF1_YEAST	1057	3171	<i>Saccharomyces cerevisiae</i>	201
SIP4_YEAST	829	2487	<i>Saccharomyces cerevisiae</i>	203
STB4_YEAST	949	2847	<i>Saccharomyces cerevisiae</i>	205
STB5_YEAST	743	2229	<i>Saccharomyces cerevisiae</i>	207
SUC1_Ca	501	1503	<i>Candida albicans</i>	209
SUT1_YEAST	299	897	<i>Saccharomyces cerevisiae</i>	211
TamA_An	739	2217	<i>Aspergillus nidulans</i>	213
TBS1_YEAST	1094	3282	<i>Saccharomyces cerevisiae</i>	215
TEA1_YEAST	759	2277	<i>Saccharomyces cerevisiae</i>	217
THI2_YEAST	450	1350	<i>Saccharomyces cerevisiae</i>	219
UAY_An	1060	3180	<i>Aspergillus nidulans</i>	221
UGA3_YEAST	528	1584	<i>Saccharomyces cerevisiae</i>	223
xlnR_Anig	875	2625	<i>Aspergillus niger</i>	225
YAKB_Sp	782	2346	<i>Schizosaccharomyces</i>	227

YAO7_Sp	637	1911	<i>pombe</i> <i>Schizosaccharomyces</i>	229
YAOc_Sp	357	1071	<i>pombe</i> <i>Schizosaccharomyces</i>	231
YAOClong_Sp	644	1932	<i>pombe</i> <i>Schizosaccharomyces</i>	233
YAS8_Sp	563	1689	<i>pombe</i> <i>Schizosaccharomyces</i>	235
YBR033W_YEA ST	919	2757	<i>pombe</i> <i>Saccharomyces</i> <i>cerevisiae</i>	237
YBR239C_YEAS T	529	1587	<i>Saccharomyces</i> <i>cerevisiae</i>	239
YCR106W_YEA ST	832	2496	<i>Saccharomyces</i> <i>cerevisiae</i>	241
YDR213W_YEA ST	913	2739	<i>Saccharomyces</i> <i>cerevisiae</i>	243
YDR303C_YEA ST	885	2655	<i>Saccharomyces</i> <i>cerevisiae</i>	245
YDR421W_YEA ST	950	2850	<i>Saccharomyces</i> <i>cerevisiae</i>	247
YDR520C_YEA ST	772	2316	<i>Saccharomyces</i> <i>cerevisiae</i>	249
YER184C_YEAS T	794	2382	<i>Saccharomyces</i> <i>cerevisiae</i>	251
YFL052W_YEA ST	465	1395	<i>Saccharomyces</i> <i>cerevisiae</i>	253
YIL130W_YEAS T	964	2892	<i>Saccharomyces</i> <i>cerevisiae</i>	255
YJL103C_YEAS T	618	1854	<i>Saccharomyces</i> <i>cerevisiae</i>	257
YJL206C_YEAS T	758	2274	<i>Saccharomyces</i> <i>cerevisiae</i>	259
YKL222C_YEAS T	705	2115	<i>Saccharomyces</i> <i>cerevisiae</i>	261
YKR064W_YEA ST	863	2589	<i>Saccharomyces</i> <i>cerevisiae</i>	263
YLL054C_YEAS T	769	2307	<i>Saccharomyces</i> <i>cerevisiae</i>	265
YLR266C_YEAS T	701	2103	<i>Saccharomyces</i> <i>cerevisiae</i>	267
YLR278C_YEAS T	1341	4023	<i>Saccharomyces</i> <i>cerevisiae</i>	269
YML076C_YEA ST	944	2832	<i>Saccharomyces</i> <i>cerevisiae</i>	271
YNR063W_YEA ST	607	1821	<i>Saccharomyces</i> <i>cerevisiae</i>	273
YOR172W_YEA	786	2358	<i>Saccharomyces</i>	275

ST			<i>cerevisiae</i>	
YOR380W_YEA	546	1638	<i>Saccharomyces</i>	277
ST			<i>cerevisiae</i>	
YPL133C_YEAS	446	1338	<i>Saccharomyces</i>	279
T			<i>cerevisiae</i>	
YPR009W_YEA	268	804	<i>Saccharomyces</i>	281
ST			<i>cerevisiae</i>	
YPR196W_YEA	470	1410	<i>Saccharomyces</i>	283
ST			<i>cerevisiae</i>	
YRR1_YEAST	810	2430	<i>Saccharomyces</i>	285
			<i>cerevisiae</i>	
ZNF1_Ca	388	1164	<i>Candida albicans</i>	287
CAB57441_Sp	497	1491	<i>Schizosaccharomyces</i>	289
			<i>pombe</i>	
PDR1SGD_YEA	1068	3204	<i>Saccharomyces</i>	291
ST			<i>cerevisiae</i>	
PDR1_YEAST	1063	3189	<i>Saccharomyces</i>	293
			<i>cerevisiae</i>	
YHL6_YEAST	883	2649	<i>Saccharomyces</i>	295
			<i>cerevisiae</i>	
At233	309	927	<i>Aspergillus terreus</i>	297
Pc1001	859	2577	<i>Penicillium</i>	299
			<i>chrysogenum</i>	
At274	424	1272	<i>Aspergillus terreus</i>	301
At221	850	2550	<i>Aspergillus terreus</i>	303
An1000	758	2274	<i>Aspergillus nidulans</i>	305
At240	576	1728	<i>Aspergillus terreus</i>	307

A "fungal homolog" of a reference gene is a fungal gene encoding a product that is capable of performing at least a portion of the function of the product encoded by the reference gene, which is substantially identical to the reference gene, and/or which encodes a product which is substantially identical to the product encoded by the reference gene. "Substantially identical" means a polypeptide or nucleic acid exhibiting at least 25%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% identity to a reference amino acid sequence or nucleic acid sequence. For polypeptides, the length of comparison sequences is generally at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids or greater. For nucleic acids, the length of comparison sequences is generally at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides or greater. Sequence identity is typically measured using sequence

analysis software (for example, the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison WI 53705; or the BLAST, BEAUTY, or PILEUP/PRETTYBOX programs). For determining percentages of identity, a gap existence penalty of 11 and a gap extension penalty of 1 may be employed in such programs. For determining sequence similarity, such software assigns degrees of homology to various substitutions, deletions, and/or other modifications.

Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

For this aspect of the invention, when the secondary metabolite is aflatoxin or sterigmatocystin, the ZBC gene is not the *aflR* gene from *Aspergillus* spp.; and when the secondary metabolite is lovastatin, the ZBC gene is not the *Aspergillus terreus* *lovE* gene.

The term "ZBC gene or gene variant" means any ZBC gene, or any useful mutant form of such gene. Many useful mutations of ZBC genes and/or proteins are contemplated by the present invention, including various dominant mutations. A "dominant mutation" is an allele of a gene that encodes a protein capable of changing the phenotype of an organism more than a non-mutated form of the gene. Preferred dominant mutations include dominant negative mutations, dominant positive mutations, and dominant neomorphic mutations. A "dominant negative mutation" is a dominant mutation that achieves its phenotypic effect by interfering with some function of the gene or gene product from which it was derived, or from a homolog thereof. A "dominant positive mutation" is a dominant mutation that achieves its phenotypic effect by activating some function of the gene or gene product from which it was derived, or from a homolog thereof. A "dominant neomorphic mutation" is a dominant mutation that achieves the phenotypic effect of providing a novel function to the gene or gene product from which it was derived, or from a homolog thereof. Preferred dominant mutations according to this aspect of the invention include:

- (1) Mutations that result in increased or decreased stability of the transcript of a gene.

(2) Mutations that result in increased or decreased stability of the product of translation: For example, specific sequences near the amino terminus of a protein have been shown to cause increased or decreased protein stability. Similarly, sequences elsewhere in the protein, such as those required for ubiquitin-dependent degradation, can be mutated to increase the stability of a protein.

(3) Amino acid substitutions that mimic post-translational modifications: For example, phosphorylation has been demonstrated to positively or negatively regulate the activity of a variety of proteins, including transcription factors and kinases. Phosphorylation most commonly occurs on serine, threonine, and tyrosine residues; in some instances residues such as aspartate and histidine can be phosphorylated. Mutations that mimic constitutive dephosphorylation can be produced by mutating the coding sequence of the phosphorylated residue to the coding sequence of an amino acid that cannot be phosphorylated and does not have a negatively charged side chain (e.g., alanine). Alternatively, substitutions that result in changing serine, threonine, or tyrosine residues to charged amino acids such as glutamate or aspartate can result in an allele that mimics constitutive phosphorylation.

Proteolytic cleavage is another post-translational mechanism for regulating the activity of a protein. Mutations that result in truncation of a protein can mimic activation by proteolysis. Mutations that change amino acids required for proteolysis can activate proteins that are negatively regulated by proteolysis.

(4) Amino acid substitutions that promote or inhibit the binding of small molecules such as ATP, cAMP, GTP or GDP: For example, Nucleotides are co-factors for many enzymes, and the nucleotide-binding domains of such proteins are highly conserved. Lysine to arginine substitutions in the nucleotide binding domain frequently result in the inhibition of enzymatic activity. Enzymatically inactive proteins can be dominant inactive molecules, acting by sequestering substrates from functional enzymes.



(5) Mutations in portions of genes that encode regulatory domains of proteins: For example, many proteins, including kinases, contain regulatory domains that function as mechanisms to control the timing of activation. Mutations in these domains might result in constitutive activation. Regulatory domains include linker regions and C terminal regions in the case of some ZBC proteins.

(6) Mutations that create a new protein function: For example, a mutation in a ZBC protein could result in altered DNA recognition specificity, such that the mutated ZBC protein can modulate the activity of pathways that it does not usually regulate.

(7) Fusion of the ZBC protein or variants thereof to a transcriptional activation domain: Transcriptional activation domains (TADS) are defined as discrete regions of proteins that promote gene expression by a variety of mechanisms that ultimately result in the activation of RNA polymerase. A TAD generally is defined as the minimal motif that activates transcription when fused to a DNA-binding domain (Webster *et al.* (1988), *Cell* 52: 169-178; Fischer *et al.* (1988), *Nature* 332: 853-856; Hope *et al.* (1988), *Nature* 333: 635-640).

As used for all aspects of the invention, the term "modulating the expression of a gene" means affecting the function of a gene's product, preferably by increasing or decreasing protein activity or creating a new protein activity through mutation; increasing or decreasing transcription; increasing or decreasing translation; increasing, decreasing or changing post-translational modification; altering intracellular localization; increasing or decreasing translocation; increasing or decreasing protein activity by fusion or by interaction of the protein with another molecule; and/or creating a new protein activity by interaction of the protein with another molecule. In some cases, such modulation is achieved by allowing or causing the expression of an exogenously supplied nucleic acid or gene, *e.g.*, by transformation. In some cases, other exogenously supplied molecules can mediate the modulation. The modulation is not achieved, however, by simply randomly mutagenizing the fungus, either spontaneously or by chemical means. In certain embodiments, the ZBC gene is from

an organism in which it is not present within a biosynthetic cluster, or the ZBC gene is not present in the biosynthetic cluster of the desired secondary metabolite to be regulated. In certain embodiments, the ZBC gene is from an organism other than the production fungus, preferably from a different species or genus. In certain embodiments, the ZBC gene in its native locus regulates a different secondary metabolite than the desired secondary metabolite produced by the production fungus. In certain embodiments, the ZBC gene in its native locus does not regulate secondary metabolism. "Native locus" means the chromosomal locus in the original organism from which the gene was cloned.

As used for all aspects of the invention, "mutation" means an alteration in DNA sequence, either by site-directed or random mutagenesis. Mutation encompasses point mutations as well as insertions, deletions, or rearrangements.

As used for all aspects of the invention, "mutant" means an organism containing one or more mutations.

In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the gene. "Over-expression of the gene" means transcription and/or translation and/or gene product maturation at a rate that exceeds by at least two-fold, preferably at least five-fold, and more preferably at least ten-fold, the level of such expression that would be present under similar growth conditions in the absence of the modulation of expression of the gene. "Similar growth conditions" means similar sources of nutrients such as carbon, nitrogen, and phosphate, as well as similar pH, partial oxygen pressure, temperature, concentration of drugs or other small molecules, and a similar substrate for growth, whether solid, semi-solid, or liquid.

In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before.

In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. "Conditional expression" of a gene means expression under certain growth conditions, but not under others. Such growth conditions that may be varied include, without limitation,

carbon source, nitrogen source, phosphate source, pH, temperature, partial oxygen pressure, the presence or absence of small molecules such as drugs, and the presence or absence of a solid substrate.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-bacterial. An "anti-bacterial" is a molecule that has cytotoxic or cytostatic activity against some or all bacteria. Preferred anti-bacterials include, without limitation,  $\beta$ -lactams. Preferred  $\beta$ -lactams include, without limitation, penicillins and cephalosporins. Preferred penicillins and biosynthetic intermediates include, without limitation, isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V. Preferred cephalosporins and biosynthetic intermediates include, without limitation, deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxy-cephalosporanic acid (7-ADCA), cephalosporin C, 7- $\beta$ -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- $\beta$ -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-hypercholesterolemic. An "anti-hypercholesterolemic" is a drug administered to a patient diagnosed with elevated cholesterol levels, for the purpose of lowering the cholesterol levels. Preferred anti-hypercholesterolemic include, without limitation, lovastatin, mevastatin, simvastatin, and pravastatin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an immunosuppressant. An "immunosuppressant" is a molecule that reduces or eliminates an immune response in a host when the host is challenged with an immunogenic molecule, including immunogenic molecules present on transplanted organs, tissues or cells. Preferred immunosuppressants include, without limitation, members of the cyclosporin family and beauverolide L. Preferred cyclosporins include, without limitation, cyclosporin A and cyclosporin C.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an ergot alkaloid. An "ergot alkaloid" is a

member of a large family of alkaloid compounds that are most often produced in the sclerotia of fungi of the genus *Claviceps*. An "alkaloid" is a small molecule that contains nitrogen and has basic pH characteristics. The classes of ergot alkaloids include clavine alkaloids, lysergic acids, lysergic acid amides, and ergot peptide alkaloids. Preferred ergot alkaloids include, without limitation, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrine, and ergoclavine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an inhibitor of angiogenesis. An "angiogenesis inhibitor" is a molecule that decreases or prevents the formation of new blood vessels. Angiogenesis inhibitors have proven effective in the treatment of several human diseases including, without limitation, cancer, rheumatoid arthritis, and diabetic retinopathy. Preferred inhibitors of angiogenesis include, without limitation, fumagillin and ovalicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a glucan synthase inhibitor. A "glucan synthase inhibitor" is a molecule that decreases or inhibits the production of 1,3- $\beta$ -D-glucan, a structural polymer of fungal cell walls. Glucan synthase inhibitors are a class of antifungal agents. Preferred glucan synthase inhibitors include, without limitation, echinocandin B, pneumocandin B, aculeacin A, and papulacandin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a member of the gliotoxin family of compounds. The "gliotoxin family of compounds" are related molecules of the epipolythiodioxopiperazine class. Gliotoxins display diverse biological activities, including, without limitation, antimicrobial, antifungal, antiviral, and immunomodulating activities. Preferred members of the "gliotoxin family of compounds" include, without limitation, gliotoxin and aspirochlorine.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a fungal toxin. A "fungal toxin" is a compound that causes a pathological condition in a host, either plant or animal. Fungal toxins could be mycotoxins present in food products, toxins produced by phytopathogens, toxins from poisonous mushrooms, or toxins produced by zoopathogens. Preferred

fungal toxins include, without limitation, aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a modulator of cell surface receptor signaling. As used herein, the term "cell surface receptor" means a molecule that resides at or in the plasma membrane, binds an extracellular signaling molecule, and transduces this signal to propagate a cellular response. Modulators of cell surface receptor signaling might function by one of several mechanisms including, without limitation, acting as agonists or antagonists; sequestering a molecule that interacts with a receptor, such as a ligand; or stabilizing the interaction of a receptor and a molecule with which it interacts. Preferred modulators of cell surface signaling include, without limitation, the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a plant growth regulator. A "plant growth regulator" is a molecule that controls growth and development of a plant by affecting processes that include, without limitation, division, elongation, and differentiation of cells. Preferred plant growth regulators include, without limitation, cytokinin, auxin, gibberellin, abscisic acid, and ethylene.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is a pigment. A "pigment" is a substance that imparts a characteristic color. Preferred pigments include, without limitation, melanins and carotenoids.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an insecticide. An "insecticide" is a molecule that is toxic to at least some insects. A preferred insecticide, without limitation, is nodulisporic acid.

In certain embodiments of the methods according to this aspect of the invention, the secondary metabolite is an anti-neoplastic compound. An "anti-

neoplastic" compound is a molecule that prevents or reduces tumor formation. Preferred anti-neoplastic compounds include, without limitation, taxol (paclitaxel) and related taxoids.

In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. "Purifying" means obtaining the secondary metabolite in substantially pure form. "Substantially pure" means comprising at least 90%, more preferably at least 95%, and most preferably at least 99%, of the purified composition on a dry-weight basis.

In a second aspect, the invention provides methods for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite in the fungus, the methods comprising modulating the expression of a ZBC gene or gene variant in a manner that improves the productivity of the secondary metabolite.

"Improves the productivity" means to increase the quotient of the concentration of the secondary metabolite divided by the product of the fermentor run-time multiplied by the fermentation volume multiplied by the grams of the dry cell weight of biomass ( $\text{Productivity} = \text{concentration metabolite} / (\text{time} \times \text{volume} \times \text{gDCW})$ ).

Significant advantages that might result from increasing productivity include, without limitation, a decrease in fermentor run-time, a decrease in the size of the fermentor required for production of equivalent amounts of secondary metabolite, or a decrease in the biomass required for production, which translates into decreased waste that must be handled in downstream processing. Preferably, such increased productivity is by at least ten percent, more preferably at least 50 percent, and most preferably at least two-fold.

"Modulating the expression of a ZBC gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the ZBC gene. "Over-expression of the gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of

the invention are as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In the methods according to this aspect of the invention, the term "secondary metabolite" is as used previously and preferred secondary metabolites include, without limitation, those discussed previously. In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a third aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing efflux or excretion of the secondary metabolite, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that increases efflux or excretion of the secondary metabolite.

"Increasing efflux or excretion of the secondary metabolite" means that, without lysing a fungal cell, a greater quantity of the secondary metabolite passes from the inside of the fungal cell to the outside of the fungal cell per unit time. "Outside of the fungal cell" is defined as being no longer contained wholly within the lipid bilayer of the cell and/or extractable from the cell with methods which do not release a majority of intracellular contents.

"Modulating the expression of a ZBC gene" is as used before, except that the ZBC gene can be the *Aspergillus* spp. *aflR* gene when the secondary metabolite is aflatoxin or sterigmatocystin, and the ZBC gene can be *lovE* when the secondary metabolite is lovastatin. In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the gene. "Over-expression of the gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In the methods according to this aspect of the invention, the term "secondary metabolite" is as used previously and preferred secondary metabolites include, without limitation, those discussed previously. In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a fourth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by decreasing production of side products or non-desired secondary metabolites, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that decreases production of side products or non-desired secondary metabolites.

"Decreasing production of side products or non-desired secondary metabolites" means reducing the amount of such side products or non-desired secondary metabolites that are synthesized or which are retained within the cells or the media surrounding the cells. Preferably, such reduction is at least by 25%, more preferably by at least 50%, even more preferably by at least 2-fold, and most preferably by at least 5-fold.

"Modulating the expression of a ZBC gene" is as used for the third aspect of the invention. In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the gene. "Over-expression of the gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In the methods according to this aspect of the invention, the term "secondary metabolite" is as used previously and preferred secondary metabolites include, without limitation, those discussed previously. In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.



In a fifth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that alters the characteristics of the fungus.

"Altering the characteristics" means changing the morphology or growth traits of the fungus. Preferred alterations include, without limitation, alterations that result in transition of the fungus from the hyphal to the yeast form; alterations that result in transition of the fungus from the yeast to the hyphal form; alterations that lead to more or less hyphal branching; alterations that increase or decrease flocculence, adherence, cell buoyancy, surface area of the fungus, cell wall integrity and/or stability, pellet size, vacuole formation, and/or ability to grow at higher or lower temperatures; and alterations that increase the saturating growth density of a culture or rate of pellet formation.

"Modulating the expression of a ZBC gene" is as used for the third aspect of the invention. In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the gene. "Over-expression of the gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In the methods according to this aspect of the invention, the term "secondary metabolite" is as used previously and preferred secondary metabolites include, without limitation, those discussed previously. In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a sixth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the

method comprising modulating the expression of a ZBC gene or gene variant in a manner that causes conditional lysis.

"Causing conditional lysis" means causing the fungus to grow without lysis under a first set of growth conditions and to lyse under a second and different set of conditions, which are not lytic to the unmodified fungus. In preferred embodiments, the conditions that can be altered between the first and second growth conditions include, without limitation, the source or amount of nutrients such as carbon, nitrogen, and phosphate; the source or amount of specific enzymes; the source or amount of specific components found in cell walls; the amount of salts or osmolytes; the pH of the medium; the partial oxygen pressure; temperature; and the amount of specific small molecules.

"Modulating the expression of a ZBC gene" is as used for the third aspect of the invention. In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the gene. "Over-expression of the gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In the methods according to this aspect of the invention, the term "secondary metabolite" is as used previously and preferred secondary metabolites include, without limitation, those discussed previously. In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In a seventh aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite made by the same organism, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that increases resistance to the deleterious effects of exposure to a secondary metabolite. As used herein, the phrase "increasing the resistance of the fungus to the

deleterious effects of exposure to a secondary metabolite" means to allow the fungus to survive, grow, or produce the secondary metabolite in conditions that otherwise would be toxic to the fungus or prevent the production of the secondary metabolite. In particular, the growth of a fungus that produces a secondary metabolite can be limited, in part, by the toxic effects of the secondary metabolite itself. In the absence of resistance mechanisms to protect the fungi from the toxic effects of these metabolites, decreased yields of the metabolite can be observed. For example, Alexander *et al.* (1999), *Mol. Gen. Genet.* 261: 977-84, have shown that the trichothecene efflux pump of *Fusarium sporotrichiodes*, encoded by the gene *TRI12*, is required both for high level production of, and resistance to the toxic effects of, trichothecenes produced by this fungus. Thus, modifications that increase the resistance of a fungus to a toxic secondary metabolite that it produces can increase the saturation density and extend the metabolically active lifetime of the producing fungus. In a bioreactor, such attributes will have the beneficial effect of increasing the yield and productivity of a metabolite. Regulators of secondary metabolite production whose expression can be modulated to increase resistance of a fungus to toxic metabolites can include, without limitation, transporters that promote efflux of the metabolite from cells, enzymes that alter the chemical structure of the metabolite within cells to render it non-toxic, target(s) of the metabolite that mediate its toxicity, and gene products that alter cellular processes to counteract the toxic effects of a metabolite. Additional benefits of increasing efflux of secondary metabolites include increasing the amount of metabolite available for purification from the fermentation broth and mitigation of feedback inhibition of secondary metabolism that may be mediated by the metabolite itself. Indeed, feedback inhibition of a biosynthetic pathway by a product of that pathway is well documented in many microorganisms, and this inhibition can act at the transcriptional, translational, and post-translational levels. Several well-documented examples in yeast include the transcriptional repression of lysine biosynthetic genes by lysine (Feller *et al.* (1999), *Eur. J. Biochem.* 261: 163-70), the decreased stability of both the mRNA encoding the uracil permease Fur4p and the permease itself in the presence of uracil (Seron *et al.* (1999), *J. Bacteriol.* 181: 1793-800), and the inhibition of alpha-isopropyl malate synthase, a key step in leucine

biosynthesis, by the presence of leucine (Beltzer *et al.* (1988), *J. Biol. Chem.* **263**: 368-74).

Transcription factors that regulate the expression of efflux pumps could also be used to increase efflux of a drug from a fungal cell to increase the yields of a metabolite and decrease the toxicity of the secondary metabolite in a fermentation. Such transcription factors include, but are not limited to, ZBC genes such as *PDR1*, and *PDR3* from *S. cerevisiae* and their homologs. Over-expression of each of these genes has been shown to up-regulate expression of transporters and cause increased resistance of *S. cerevisiae* to toxic compounds (for examples, see Reid *et al.* (1997), *J. Biol. Chem.* **272**: 12091-9; Katzmann *et al.* (1994), *Mol. Cell. Biol.* **14**: 4653-61; Wendler *et al.* (1997), *J. Biol. Chem.* **272**: 27091-8).

Increases in resistance to the toxic effects of secondary metabolites will vary with the metabolite. For example, amatoxins kill cells by inhibiting the function of the major cellular RNA polymerase, RNA polymerase II, in eucaryotic cells. Mutant forms of RNA polymerase II resistant to the effects of alpha-amanitin have been described (Bartolomei *et al.* (1988), *Mol. Cell. Biol.* **8**: 330-9; Chen *et al.* (1993), *Mol. Cell. Biol.* **13**: 4214-22). Similarly, mutations affecting HMG CoA reductase, the target enzyme for the secondary metabolite lovastatin, have been identified. Increased levels of HMG CoA Reductase can also cause resistance to lovastatin (Ravid *et al.* (1999), *J. Biol. Chem.* **274**: 29341-51; Lum *et al.* (1996), *Yeast* **12**: 1107-24). Taxol (paclitaxel), causes lethality by increasing microtubule stability, thus preventing exit from mitosis. Dominant mutations affecting  $\beta$ -tubulin that confer resistance to taxol have been characterized (for example, see Gonzalez *et al.* (1999), *J. Biol. Chem.* **274**: 23875-82) and could prove to be useful to confer resistance to this toxic metabolite in production strains. The pneumocandin and echinocandin families of metabolites are fungal secondary metabolites that inhibit the enzyme 1,3- $\beta$ -D-glucan synthase. Dominant mutations in the *C. albicans* glucan synthase gene, *FKS1*, have been shown to confer resistance to candins (Douglas *et al.* (1997), *Antimicrob. Agents Chemother.* **41**: 2471-9). Glucan synthase mutations such as these could be used to generate fungal production strains with increased resistance to the candin class of antifungals. *S. cerevisiae* mutants resistant to the growth-inhibitory effects of the fungal secondary metabolite cyclosporin A have also been described (Cardenas *et al.* (1995), *EMBO J.*

14: 2772-83). These mutants were shown to harbor mutations in *CNA1*, the gene encoding the catalytic subunit of the heterodimeric calcium-calmodulin dependent phosphatase, calcineurin A. Fumagillin, an antiangiogenic agent, binds to and inhibits the N-terminal aminopeptidases in a wide variety of both procaryotes and eucaryotes (Sin *et al.* (1997), *Proc. Natl. Acad. Sci. USA* 94: 6099-103, Lowther *et al.* (1998), *Proc. Natl. Acad. Sci. USA* 95: 12153-7). Mutations in this enzyme that block fumagillin binding and/or inhibitory activity could well prove useful in enhancing the resistance of fungal production strains to the growth inhibitory effects of this secondary metabolite.

"Modulating the expression of a ZBC gene" is as used for the third aspect of the invention. In certain embodiments of the methods according to this aspect of the invention, the modulation is over-expression of the gene. "Over-expression of the gene" is as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is expression of a dominant mutation of the gene. The term "dominant mutation" is as used before. Preferred dominant mutations according to this aspect of the invention are as used before. In certain embodiments of the methods according to this aspect of the invention, the modulation is conditional expression of the gene. The term "conditional expression" of a gene is as used before.

In the methods according to this aspect of the invention, the term "secondary metabolite" is as used previously and preferred secondary metabolites include, without limitation, those discussed previously. In certain embodiments of the methods according to this aspect of the invention, the methods further comprise purifying the secondary metabolite from a culture of the fungus. The term "purifying" is as used before.

In an eighth aspect, the invention provides methods for improving production of a secondary metabolite in a fungus by modulating the expression of one or more genes, the method comprising modulating the expression of a ZBC gene or gene variant that does not normally modulate the expression of such gene or genes.

In a ninth aspect, the invention provides genetically modified fungi, wherein the genetically modified fungi have an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by any of the methods according to the invention.

In a tenth aspect, the invention provides a method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to the invention under conditions suitable for the production of secondary metabolites.

Nine ZBC genes (and derivatives thereof) have been tested to date for effects on either lovastatin yield in *A. terreus* or penicillin production in *P. chrysogenum*, and in some cases both. Of these genes (*lovE*, *lovU*, *An13*, *At18*, *CAT8*, *SIP4*, *LYS14*, *tamA*, and *YAF1*) three of the nine (*lovU*, *At18* and *LYS14*), or 33%, have demonstrable positive effects on metabolite production. Details of the *lovU*, *At18* and *LYS14* results are described in the examples presented below.

The following examples illustrate some preferred modes of practicing the present invention, but are not intended to limit the scope of the claimed invention. Alternative materials and methods may be utilized to obtain similar results.

#### EXAMPLE 1

##### Construction of an Expression Vector for the *At18* Gene from *A. terreus*.

To test whether a ZBC gene that is not encoded within the biosynthetic cluster for the production of a specific metabolite can regulate the biosynthesis of that specific metabolite, the ZBC-encoding *At18* gene from *A. terreus* was tested for effects on lovastatin production. To over-express *At18* (SEQ ID NO 51) in *A. terreus*, *At18* was amplified with oligonucleotides MO1715 (SEQ ID NO 1) and MO1716 (SEQ ID NO 2) using Turbo Pfu DNA Polymerase and a cDNA clone of *At18* as a template under standard conditions for polymerase chain reaction (PCR). A GATEWAY Cloning Technology (Invitrogen Corp., Carlsbad, CA) entry vector was produced from the resultant PCR product and the GATEWAY pDONR206 entry plasmid according to manufacturer's instructions. The resultant vector, MB1754, was then reacted in a with plasmid MB1419 to form an expression vector according to manufacturer's instructions. MB1419 is derived from pLXZ161, a vector derived from pBC-phleo (Silar (1995), *Fungal Genetics Newsletter* 42: 73) that carries a phleomycin resistance cassette for selection of transformants in *A. terreus*, as well as a polylinker located between the *A. nidulans* PGK promoter and the *A. nidulans trpC* terminator. pLXZ161 is constructed as follows: First, the *A. nidulans trpC* terminator is amplified from genomic DNA by the PCR using Turbo Pfu Polymerase as

described by the manufacturer (Stratagene, La Jolla, CA). Primers used in this reaction are TRPC-1 (SEQ ID NO 3) and TRPC-2 (SEQ ID NO 4). The resultant product is digested with the restriction enzymes SacII and NotI, purified by agarose gel electrophoresis, and cloned into SacII/NotI-digested pBC-phleo DNA, to generate pLXZ116. Second, the *A. nidulans* PGK promoter is amplified from *A. nidulans* genomic DNA by PCR using primers PGK1-1 (SEQ ID NO 5) and PGK1-2 (SEQ ID NO 6), Turbo Pfu Polymerase, and the reaction conditions as described above. The resultant product is digested with ApaI and ClaI and cloned into ApaI/ClaI-digested pLXZ116, to generate pLXZ161. To produce MB1419, the *ccdB* (death gene) cassette from pEZC7201 (Invitrogen Corp., Carlsbad, CA) was amplified by PCR using oligos MO511 (SEQ ID NO 7) and MO512 (SEQ ID NO 8), digested with ClaI and NotI, and cloned into NotI/ClaI-digested pLXZ161. This generated an expression vector in which the death gene cassette resides between the *A. nidulans* PGK promoter and the *A. nidulans trpC* terminator of pLXZ161. The reactions using this vector allow configuration of any gene in an entry clone to be expressed under the control of the *A. nidulans* PGK promoter (see GATEWAY Cloning Technology manual, Invitrogen Corp., Carlsbad, CA). The fungal selectable marker contained on this plasmid is *ble*, which confers resistance to phleomycin. Reaction of MB1754 with MB1419 yielded a clone (MB1970) which is configured to express *At18* under control of the *A. nidulans* PGK promoter, with the terminator region from the *A. nidulans trpC* gene acting as a transcriptional terminator.

## EXAMPLE 2

### Transformation of *A. terreus* with a ZBC Gene

For transformation of *A. terreus*, spores were first generated by culture of strain ATCC#20542 on petri plates containing potato dextrose agar (PDA, Becton Dickinson & Co., Sparks, MO) at 30°C for 3-6 days. Spores were removed from PDA either by resuspension in sterile water or Tween-80 (0.1%) or by scraping directly from the plate using a sterile spatula. Yeast extract sucrose (YES ) medium (2% yeast extract, 6% sucrose) was inoculated to a density of  $1-5 \times 10^6$  spores per ml and incubated with shaking in an Erlenmeyer flask at 26-30° C for 12-16 hr (250 rpm). Mycelia were harvested by centrifugation at 3200 rpm for 10 minutes, and washed in

sterile water twice. Mycelia were resuspended in a filter-sterilized solution of Novozyme 234 (Sigma, St. Louis, MO) at 2-5 mg/ml in 1 M  $\text{MgSO}_4$  and digested at room temperature with shaking (80 rpm) for 1-2 hr. Undigested material was removed from the digest by filtration through a rayon-polyester cloth with acrylic binder and 22-25  $\mu\text{m}$  pores (MIRACLOTH, Calbiochem, San Diego, CA). After adding 1-2 volumes of STC (0.8 M sorbitol, 25 mM Tris, pH 7.5, and 25 mM  $\text{CaCl}_2$ ), the protoplasts were pelleted by centrifugation at 2500 rpm. Protoplasts were washed twice in STC by centrifugation. Resulting protoplasts were resuspended to a density of  $5 \times 10^7$  per ml in a solution of STC, SPTC (40% polyethylene glycol in STC) and DMSO in a ratio of 9:1:0.1 and frozen at  $-80^\circ\text{C}$ . Two aliquots (100  $\mu\text{l}$  each) of protoplasts were mixed with 1-5  $\mu\text{g}$  of either pBCphleo or MB1970 DNA and incubated on ice for 30 min. An aliquot of SPTC (15  $\mu\text{l}$ ) was added to each tube and the reaction was incubated at room temperature for 15 minutes. An additional aliquot (500  $\mu\text{l}$ ) was added with gentle mixing, and the reaction was incubated for an additional 15 minutes at room temperature. The reaction was next resuspended in 25 ml of molten regeneration medium (PDA from Sigma, St Louis, MO) with 0.8 M sucrose, maintained at  $50^\circ\text{C}$ , and poured onto a 150 mm petri plate containing 25 ml of solidified regeneration medium plus phleomycin (60-200  $\mu\text{g}/\text{ml}$ ). Transformants were typically visible after 2-5 days of incubation at  $26-30^\circ\text{C}$ .

*A. terreus* transformants were grown on modified RPM medium (WO 00/37629) containing 4% glucose, 0.3% corn steep liquor (Sigma, St. Louis, MO), 0.2%  $\text{KNO}_3$ , 0.3%  $\text{KH}_2\text{PO}_4$ , 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05%  $\text{NaCl}$ , 0.05% polyglycol (Dow Chemical Co., Midland, MI), 0.1 % trace elements (14.3 g/l  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.5 g/l  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.5 g/l  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 13.8 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 8.5 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 3 g/l citric acid  $\cdot \text{H}_2\text{O}$  (add first), 1 g/l  $\text{H}_3\text{BO}_3$ , 1 g/l  $\text{Na}_2\text{MoO}_4$ , 2.5 g/l  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ). The final pH was adjusted to 6.5. Spores for the inoculum were generated by culturing on plates containing minimal medium plus phleomycin for 1 week at 27 C. Spores for shake flask inoculation were removed from plates by dragging the tip of a sterile wooden stick approximately 1 inch across the plate surface. The tip of the stick was then dipped into the shake flask medium and swirled gently. Cultures were grown at  $27^\circ\text{C}$ , 225 RPM for 5-6 days.



### EXAMPLE 3

#### Determination of Lovastatin Production

Lovastatin is known to inhibit the enzyme HMG-CoA reductase (HMGR) which converts hydroxymethylglutaryl coenzyme A (HMGCoA) and NADPH to mevalonate and NADP. This reaction can be quantified by measuring the change in absorbance of NADPH. To assay lovastatin production, 6-histidine tagged HMGR ((His)<sub>6</sub>HMGR) was first expressed in *S. cerevisiae* and purified with a nickel column. *A. terreus* samples were fermented as described above and 0.5 mL samples were taken at day 5-6, put in a 1 mL 96-well plate and centrifuged to remove mycelia before assaying. Samples were transferred to another 1 mL 96-well plate and frozen at -80°C.

Samples were thawed and 10 µL removed and diluted 1:50 in H<sub>2</sub>O. 10µl of this diluted broth was assayed in a reaction (200 µL total) containing 1 mM L-HMGCoA, 1 mM NADPH, 0.005 mM DTT and 5 µL (His)<sub>6</sub>HMGR. The disappearance of absorbance at 340 nm was observed over time. This represented the disappearance of NADPH. Lovastatin inhibits this reaction. The initial velocities were calculated for the reactions containing samples, adjusted for dilution and compared to reactions containing lovastatin standards to determine levels of metabolite produced by regression analysis. The amounts of lovastatin produced by six *At18* transformants and six pBC-phleo (vector) transformants were determined. The median value of lovastatin production by the vector transformants was approximately 100 micrograms/mL broth whereas that of the *At18* transformants was approximately 210 micrograms/ml broth. These results demonstrate that a ZBC gene that is not encoded within the biosynthetic gene cluster of a particular secondary metabolite can nonetheless regulate the production of that metabolite.

### EXAMPLE 4

#### Construction of an Expression Vector for the *lovU* Gene from *A. terreus*.

To test *lovU* (previously designated as *orf13* in *A. terreus*, Genbank ID 4959954) function in *P. chrysogenum*, *lovU* was amplified by PCR under standard conditions well known to those in the art, using *A. terreus* first strand cDNA as template. To generate first strand cDNA, *A. terreus* (MF22; ATCC#20542) was

grown for 45 hours in Production Media (Cerelose, 4.5% (w/v), Peptonized Milk, 2.5% (w/v), Autolyzed yeast, 0.25% (w/v), Polyglycol P2000, 0.25% (w/v), pH to 7.0) at 25°C. Mycelia were harvested in a 50 cc syringe plugged with sterile cotton wool using a vacuum apparatus, washed once with sterile H<sub>2</sub>O, and snap frozen in liquid nitrogen. Mycelia were then ground to a powder under liquid nitrogen with a mortar and pestle, and homogenized in RLC buffer (RNeasy Kit; Qiagen, Inc., Valencia, CA) using a GLH rotor-stator homogenizer (Omni International, Warrenton, VA.) Total RNA was purified using an RNeasy Maxi column according to the instructions of the manufacturer.

The polyA+ fraction of the *A. terreus* total RNA was isolated using Oligotex beads (Qiagen, Inc., Valencia, CA). Purified polyA+ RNA (5 µg) was used to generate complementary DNA (cDNA) using SUPERScript reverse transcriptase (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. First strand cDNA was used to amplify cDNA predicted to encode *lovU* using PCR. Oligos used to amplify the *lovU* cDNA were MO843 (SEQ ID NO 9) and MO844 (SEQ ID NO 10). The resultant *lovU* PCR product was cloned using GATEWAY Cloning Technology (Invitrogen Corp., Carlsbad, CA) to generate the entry clone MB1201. This vector was then used to generate the expression vector MB1317 which encodes *lovU* under control of the *A. nidulans* PGK promoter. The dominant selectable marker for transformation on this vector is the *ble* gene under control of the *A. nidulans* GPD promoter, which confers resistance to the antimicrobial agent phleomycin.

#### EXAMPLE 5

##### Transformation of *P. chrysogenum* Strains MF1 and MF20

MB1325 (a control plasmid expressing the *ble* marker gene which causes phleomycin resistance) and MB1317 were transformed into *P. chrysogenum* strains MF1 (NRRL1951) and MF20 (ATCC 11702). Transformation was accomplished in the same manner as described above for *A. terreus*, except transformants were selected on 30 µg/mL phleomycin. To test levels of penicillin produced in *P. chrysogenum* transformants, a plug containing spores and mycelia was used as the inoculum. The medium used was the published P2 production medium (Lein (1986), in *Overproduction of Microbial Metabolites*, pp. 105-139, Vanek and Hostalek (eds.),

Butterworth Heinemann, Woburn, MA) which contains, 30% lactose, 5X pharmamedia cotton seed flour, ammonium sulfate, calcium carbonate, potassium phosphate, potassium sulfate, and phenoxyacetic acid, at pH 7. Flasks were incubated at 26°C with shaking at 225 rpm.

Sampling was done after 6 days of growth. 1-1.5 ml of supernatant is placed into 96-well plates. Plates were centrifuged and supernatants transferred to a new 96-well plate. Standard samples contained 0, 25, 50, 100, 200, 300, 400, and 500 µg/mL phenoxymethylpenicillin (sodium salt) dissolved in 10 mM potassium phosphate (pH 7.0), and assays were conducted as described below.

### EXAMPLE 6

#### Determination of Penicillin Production

Fermentation broth was clarified by centrifugation for 10 min at 4000 g, and 40 µL of clarified fermentation broth and penicillin standard solutions was pipetted into individual wells of a 96-well UV collection plate. Next, 200 µL of imidazole reagent was pipetted into a 96-well filter plate (0.45 micron). The derivatization reaction of penicillin was initiated by vacuum filtration of imidazole reagent into a collection plate containing the aliquoted samples and standards. The collection plate was placed into a 96-well plate reader at 45 degrees while absorbance at 325 nm was monitored over 20 minutes. A Molecular Dynamics (Sunnyvale, CA) 96-well UV/Vis plate reader was used for all spectrophotometric detection. A 1.2 M aqueous imidazole solution containing mercuric chloride at a concentration of 1 mM, pH 6.8 was prepared as follows: 8.25 g of imidazole was dissolved in 60 mL of water, 10 mL of 5 M HCl was added, and then 10 mL of a solution of mercuric chloride (0.27 g dissolved in 100 mL of water) was added. The pH was adjusted to 6.80 +/- 0.05 with 5 M HCl and the volume was brought to 100 mL with water (see, *e.g.*, Bundgaard and Iler (1972), *Journal of Pharm. Pharmac.* 24: 790-794). Results for the effect of *lovU* on penicillin production in MF1 are shown in Fig. 2, and the results for the effect of *lovU* in MF20 are shown in Fig. 3. These results demonstrate that ZBC genes from one fungal genus can regulate a biosynthetic cluster or secondary metabolite production in a fungus from a different genus.

### EXAMPLE 7

#### Effect of *At18* on Lovastatin Productivity

The *At18* construct according to Example 1 is used to transform *A. terreus* according to Example 2. Lovastatin concentration is determined according to Example 3, except that it is measured as the amount of lovastatin per unit volume of broth per gram of dry cell weight at each 24 hour time point of the fermentation and expressed as concentration as a function of fermentation time. Transformants with increased productivity, relative to vector controls, have a higher concentration of lovastatin at earlier time points.

### EXAMPLE 8

#### Effect of *At18* on Undesired Metabolites

The *At18* construct according to Example 1 is used to transform *A. terreus* according to Example 2. Transformants are grown in production media and sampled at six and twelve days. The concentration of the undesired polyketide metabolite sulochrin is measured in samples of the whole fermentation broth (broth plus cell mass) at various time points for every sample. For each whole broth sample, the pH is adjusted to 7.7 and an equal volume of methanol is added. These whole broth/methanol extracts are assessed for the concentration of sulochrin at each time point using HPLC analysis. Conditions for determining sulochrin concentrations by HPLC are standard (see, e.g., Vinci *et al.* (1991), *J. Ind. Microbiol.* 8: 113-120) and involve separation on a C-8 HPLC column using a mobile phase of 0.1% H<sub>3</sub>PO<sub>4</sub>-acetonitrile (40:60, v/v) and measurement of absorbance by sulochrin at 238 nm (see, e.g., Schimmel and Parsons (1999), *Biotechnology Techniques* 13:379-384.)

### EXAMPLE 9

#### Construction of an Expression Vector for the *LYS14* Gene from *S. cerevisiae*.

To test *S. cerevisiae* *LYS14* function in *A. terreus*, *LYS14* was amplified by PCR under standard conditions from *S. cerevisiae* genomic library DNA using primers MO931 (SEQ ID NO 11) and MO932 (SEQ ID NO 12). The resultant 2.4 kb PCR product was cloned using GATEWAY Cloning Technology (Invitrogen Corp., Carlsbad, CA) to generate entry clone MB1567. Using methods well known to those experienced in

the art, MB1567 was used to generate filamentous fungal expression plasmids MB1669, MB3130, and MB3139 which encode *LYS14* under the control of, respectively, the *A. nidulans* PGK (MB1669 and MB3130) and *A. nidulans* GPD (MB3139) promoters. The dominant selectable marker for transformation on MB1669 is the *ble* gene under control of the *A. nidulans* GPD promoter, which confers resistance to the antimicrobial agent phleomycin. MB3130 and MB3139 contain the *ble* gene under control of the *A. nidulans* *trpC* promoter. Corresponding vector-only controls for the *LYS14* expression plasmids are MB2143 (GPD-*ble*) and MB2941 (*trpC-ble*).

#### EXAMPLE 9

##### Determination of Lovastatin Production.

100  $\mu$ L of broth sample was removed and diluted 1:10 into 70% H<sub>2</sub>O-30% acetonitrile (900  $\mu$ L). This mixture was spun down to pellet debris at 13000 rpm for 5 minutes. 900  $\mu$ L of this diluted broth was transferred to a vial and the sample was analyzed by HPLC. 10  $\mu$ L were injected into a Waters HPLC system (996 photo-diode array detector, 600 E pump controller and 717 autosampler) equipped with a YMC-Pack ODS column (Aq-302-3, 150 x 4.6 mm ID, S-3  $\mu$ M pore size) and eluted with isocratic 40% aqueous acetic acid (0.7%)-60% acetonitrile for 8 minutes. Lovastatin was detected at 238 nm, found to have a retention time of 6.5 minutes, and was quantitated using a calibration curve created from pure lovastatin samples.

#### EXAMPLE 10

##### Effect of *LYS14* on lovastatin production.

The *LYS14* constructs according to Example 8 was used to transform *A. terreus* according to the method of Example 2. Lovastatin concentration was determined according to the methods of Example 9.

Thus, *A. terreus* strain MF22 was transformed with MB2143 (GPD-*ble* vector control), MB1669 (PGK-*LYS14*, GPD-*ble*), MB2941 (*trpC-ble* vector control), MB3130 (PGK-*LYS14*, *trpC-ble*) and MB3139 (GPD-*LYS14*, *trpC-ble*). Lovastatin concentration from the corresponding vector-only was used as a control. For example, MB2143 was the vector control for MB1669. The median value and highest data

point for lovastatin production of the *LYS14* expression plasmids MB1669 and MB3139 was greater than that of the corresponding vector only controls. In contrast, the median value for MB3130 (PGK-*LYS14*, *trpC-ble*) was lower than that of the corresponding vector-only control, suggesting that the different properties of the expression vector can influence ability of the construct to integrate, the locus of integration, copy number, etc. Similar results, in which some vectors work better than others, have been observed for other genes. Therefore, one of skill in the art can test more than one vector to optimize results.

#### Equivalents.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the appended claims.

What is claimed is:

1. A method for improving the production of a secondary metabolite by a fungus by increasing the yield of the secondary metabolite produced by the fungus, the method comprising:

modulation of the expression of at least one ZBC gene in a manner that improves the yield of the secondary metabolite;

wherein, the ZBC gene is not *aflR* if the secondary metabolite is aflatoxin or sterigmatocystin; and

wherein the ZBC gene is not *lovE* if the secondary metabolite is lovastatin.

2. A method as in claim 1, wherein said ZBC gene comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOS 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, and 307, and fungal homologs thereof.

3. A method as in claim 1, wherein said modulation of said ZBC gene expression results from transformation of said fungus with an exogenously supplied nucleic acid encoding said ZBC gene.

4. A method as in claim 1, wherein the modulation of said ZBC gene is over-expression of the ZBC gene.

5. A method as in claim 1, wherein the modulation of the ZBC gene is conditional expression of the ZBC gene.
6. A method as in claim 1, wherein the secondary metabolite is an antibacterial.
7. A method as in claim 6, wherein the antibacterial is a  $\beta$ -lactam.
8. A method as in claim 7, wherein the  $\beta$ -lactam is selected from the group consisting of penicillins and cephalosporins.
9. A method as in claim 8, wherein the penicillin is selected from the group consisting of isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V.
10. A method as in claim 8, wherein the cephalosporin is selected from the group consisting of deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7-  $\beta$ -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7-  $\beta$ -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).
11. A method as in claim 1, wherein the secondary metabolite is an anti-hypercholesterolemic.
12. A method as in claim 11, wherein the anti-hypercholesterolemic is selected from the group consisting of lovastatin, mevastatin, simvastatin, and pravastatin.
13. A method as in claim 1, wherein the secondary metabolite is an immunosuppressant.
14. A method as in claim 13 wherein the immunosuppressant is selected from the group consisting of members of the cyclosporin family and beauverolide L.



15. A method as in claim 14 wherein the cyclosporin is selected from the group consisting of cyclosporin A and cyclosporin C.
16. A method as in claim 1, wherein the secondary metabolite is an ergot alkaloid.
17. A method as in claim 16, wherein the ergot alkaloid is selected from the group consisting of clavine alkaloids, lysergic acids, lysergic acid amides, ergot peptide alkaloids, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocominine, ergonovine, ergometrinine, and ergoclavine.
18. A method as in claim 1, wherein the secondary metabolite is an inhibitor of angiogenesis.
19. A method as in claim 18, wherein the inhibitor of angiogenesis is selected from the group consisting of fumagillin and ovalicin.
20. A method as in claim 1, wherein the secondary metabolite is a glucan synthase inhibitor.
21. A method as in claim 20, wherein the glucan synthase inhibitor is selected from the group consisting of echinocandin B, pneumocandin B, aculeacin A, and papulacandin.
22. A method as in claim 1, wherein the secondary metabolite is a member of the gliotoxin family of compounds.
23. A method as in claim 22, wherein the member is selected from gliotoxin and aspirochlorine.
24. A method as in claim 1, wherein the secondary metabolite is a fungal toxin.

25. A method as in claim 24, wherein the fungal toxin is selected from the group consisting of aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.
26. A method as in claim 1, wherein the secondary metabolite is a modulator of cell surface receptor signaling.
27. A method as in claim 26, wherein the modulator of cell surface receptor signaling is selected from the group consisting of the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.
28. A method as in claim 1, wherein the secondary metabolite is a plant growth regulator.
29. A method as in claim 28, wherein the plant growth regulator is selected from the group consisting of cytokinin, auxin, gibberellin, abscisic acid, and ethylene.
30. A method as in claim 1, wherein the secondary metabolite is a pigment.
31. A method as in claim 30 wherein the pigment is selected from the group consisting of melanins and carotenoids.
32. A method as in claim 1, wherein the secondary metabolite is an insecticide.
33. A method as in claim 32, wherein the insecticide is nodulisporic acid.
34. A method as in claim 1, wherein the secondary metabolite is an anti-neoplastic compound.

35. A method as in claim 34, wherein the antineoplastic compound is selected from taxol (paclitaxel) and related taxoids.

36. A method for improving production of a secondary metabolite by a fungus by increasing productivity of the secondary metabolite by the fungus, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that improves the productivity of the secondary metabolite, provided however that when the secondary metabolite is aflatoxin, then the ZBC gene is not aflR, and when the secondary metabolite is lovastatin, then the ZBC gene is not lovE.

37. The method according to claim 36, wherein one or more ZBC genes are selected from the group consisting of the genes corresponding to SEQ ID NOS 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, and 307, and fungal homologs thereof.

38. The method according to claim 36, wherein the modulation of the ZBC gene results from it being exogenously supplied to the fungus.

39. The method according to claim 36, wherein the modulation of the ZBC gene is over-expression of the ZBC gene.

40. The method according to claim 36, wherein the modulation of the ZBC gene is conditional expression of the ZBC gene.

41. The method according to claim 36, wherein the secondary metabolite is an antibacterial.
42. The method according to claim 41, wherein the antibacterial is a  $\beta$ -lactam.
43. The method according to claim 42, wherein the  $\beta$ -lactam is selected from the group consisting of penicillins and cephalosporins.
44. The method according to claim 43, wherein the penicillin is selected from the group consisting of isopenicillin N, 6-aminopenicillanic acid (6-APA), penicillin G, penicillin N, and penicillin V.
45. The method according to claim 43, wherein the cephalosporin is selected from the group consisting of deacetoxycephalosporin V (DAOC V), deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC), 7-aminodeacetoxycephalosporanic acid (7-ADCA), cephalosporin C, 7- $\beta$ -(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7ACA), 7- $\beta$ -(4-carboxybutanamido)-cephalosporanic acid (GL-7ACA), and 7-aminocephalosporanic acid (7ACA).
46. The method according to claim 36, wherein the secondary metabolite is an anti-hypercholesterolemic.
47. The method according to claim 46, wherein the anti-hypercholesterolemic is selected from the group consisting of lovastatin, mevastatin, simvastatin, and pravastatin.
48. The method according to claim 36, wherein the secondary metabolite is an immunosuppressant.
49. The method according to claim 48 wherein the immunosuppressant is selected from the group consisting of members of the cyclosporin family and beauverolide L.

50. The method according to claim 49 wherein the cyclosporin is selected from the group consisting of cyclosporin A and cyclosporin C.
51. The method according to claim 36, wherein the secondary metabolite is an ergot alkaloid.
52. The method according to claim 51, wherein the ergot alkaloid is selected from the group consisting of clavine alkaloids, lysergic acids, lysergic acid amides, ergot peptide alkaloids, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, ergocorninine, ergonovine, ergometrinine, and ergoclavine.
53. The method according to claim 36, wherein the secondary metabolite is an inhibitor of angiogenesis.
54. The method according to claim 53, wherein the inhibitor of angiogenesis is selected from the group consisting of fumagillin and ovalicin.
55. The method according to claim 36, wherein the secondary metabolite is a glucan synthase inhibitor.
56. The method according to claim 55, wherein the glucan synthase inhibitor is selected from the group consisting of echinocandin B, pneumocandin B, aculeacin A, and papulacandin.
57. The method according to claim 36, wherein the secondary metabolite is a member of the gliotoxin family of compounds.
58. The method according to claim 57, wherein the member is selected from gliotoxin and aspirochlorine.

59. The method according to claim 36, wherein the secondary metabolite is a fungal toxin.
60. The method according to claim 59, wherein the fungal toxin is selected from the group consisting of aflatoxins, patulin, zearalenone, cytochalasin, griseofulvin, ergochrome, cercosporin, marticin, xanthocillin, coumarins, tricothecenes, fusidanes, sesterpenes, amatoxins, malformin A, phallotoxins, pentoxin, HC toxin, psilocybin, bufotenine, lysergic acid, sporodesmin, pulcheriminic acid, sordarins, fumonisins, ochratoxin A, and fusaric acid.
61. The method according to claim 36, wherein the secondary metabolite is a modulator of cell surface receptor signaling.
62. The method according to claim 61, wherein the modulator of cell surface receptor signaling is selected from the group consisting of the insulin receptor agonist L-783,281 and the cholecystokinin receptor antagonist asperlicin.
63. The method according to claim 36, wherein the secondary metabolite is a plant growth regulator.
64. The method according to claim 63, wherein the plant growth regulator is selected from the group consisting of cytokinin, auxin, gibberellin, abscisic acid, and ethylene.
65. The method according to claim 36, wherein the secondary metabolite is a pigment.
66. The method according to claim 65, wherein the pigment is selected from a modified fungus.
67. The method according to claim 30 wherein the pigment is selected from the group consisting of melanins and carotenoids.

68. The method according to claim 1, wherein the secondary metabolite is an insecticide.
69. The method according to claim 32, wherein the insecticide is nodulisporic acid.
70. The method according to claim 1, wherein the secondary metabolite is an anti-neoplastic compound.
71. The method according to claim 34, wherein the antineoplastic compound is selected from taxol (paclitaxel) and related taxoids.
72. A method for improving production of a secondary metabolite in a fungus by decreasing production of side products or non-desired secondary metabolites, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that decreases production of side products or non-desired secondary metabolites.
73. A method for improving production of a secondary metabolite in a fungus by altering the characteristics of the fungus in a manner that is beneficial to the production of the secondary metabolite, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that alters the characteristics of the fungus.
74. A method for improving production of a secondary metabolite in a fungus by causing conditional lysis of the fungus, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that causes conditional lysis.
75. A method for improving production of a secondary metabolite in a fungus by increasing the resistance of the fungus to the deleterious effects of exposure to a secondary metabolite made by the same organism, the method comprising modulating the expression of a ZBC gene or gene variant in a manner that increases resistance to the deleterious effects of exposure to the secondary metabolite.

76. A method for improving production of a secondary metabolite in a fungus by modulating the expression of one or more genes, the method comprising modulating the expression of a ZBC gene or gene variant that does not normally modulate the expression of such gene or genes.

77. A genetically modified fungus, wherein the genetically modified fungus has an ability to produce secondary metabolites and the ability of the genetically modified fungus to produce secondary metabolites has been improved by the method according to any of claims 1-76 or 78-83.

78. A method for making a secondary metabolite, the method comprising culturing a genetically modified fungus according to claim 77 under conditions suitable for the production of secondary metabolites.

79. The method according to any of claims 1-35, wherein the ZBC gene is from an organism in which it is not present within a biosynthetic cluster of a secondary metabolite.

80. The method according to any of claims 1-35, wherein the ZBC gene is from an organism other than the fungus in which the secondary metabolite is produced.

81. The method according to any of claims 1-35, wherein the ZBC gene in its native locus regulates a different secondary metabolite than the desired secondary metabolite produced by the fungus.

82. The method according to any of claims 1-35, wherein the ZBC gene in its native locus does not regulate secondary metabolism.

83. The method according to claim 1, wherein the gene variant is a dominant mutation.



84. The method according to claim 36, wherein the gene variant is a dominant mutation.

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